LIPID A REMOVAL BY A REDUCED PRESSURE NITROGEN AFTERGLOW

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ABSTRACT

The bactericidal efficiency of a pure nitrogen flowing afterglow at reduced pressure ($1 - 20\ Torr$) has recently be demonstrated. In the first part of this paper, it is shown that this efficiency is essentially due to the N-atoms present in the late afterglow region and not to the remaining UV radiation. In the second part, the same afterglow is used on pure lipid A (P-LA) and on lipid A extracted from exposed bacteria (B-LA). In both cases, more than 60% of the exposed lipid A are removed after $40\ min$ of afterglow exposure. This decreases also results in a loss of the lipid A proinflammatory activity, assessed by the net decrease of the nuclear translocation of the redox-sensitive *NF-κB* transcription factor in murine aortic endothelial cells.

1. INTRODUCTION

Lipo-polysaccharides LPS are constituents of gram negative bacteria membranes liberated in large amounts during bacterial death; they are requiring temperatures of about $200-250\,^{\circ}\mathrm{C}$ during 30 to 60 min to be destroyed, much higher than the one used by conventional sterilization means [1, 2]. The inactivation efficiency of a pure nitrogen afterglow was recently established, demonstrating the synergistic effect existing between the treatment temperature and the concentration of the nitrogen atoms present in the afterglow [3]. New sets of experiments are presented in order to clarify the respective roles of the N-atoms and of the the UV radiation in the inactivation mechanisms by the nitrogen afterglow. The ability of the nitrogen late afterglow to remove the lipid A (a pyrogenic pro-inflammatory component of LPS, [4]) is also studied. Finally, the loss of pro-inflammatory activity of lipid A, assessed by the net decrease of the nuclear translocation of *NF-κB*, a classical LPS target, is established [5].

2. MATERIAL AND METHODS

2.a. Nitrogen afterglow system

The plasma device used in this study (Fig.1) is made of a cylindrical Pyrex reactor ($5\ litre$) filled with pure nitrogen at controlled flow-rate in the range between ($Q_{N_2} = 0.1 - 3.0\ slpm$). The discharge is created in a quartz tube of internal diameter $4\ mm$ by a $2.45\ GHz$ microwave generator (*surfatron*) with an injected power P_{MW} varying between 50 and 300 W. The total distance d between the surfatron and the reactor is set to $56\ cm$. At $15\ cm$ to the surfatron exit, the discharge tube enlarges to $19\ mm$ before a bent (to minimize the total size of the system and to prevent the UV radiation to reach the samples in the reactor) and is connected by a Teflon junction to the Pyrex rector. The pressure, measured in the reactor by a pressure gauge, can be varied between 4 and 30 $Torr$ with a throttle valve coupled to a primary vacuum pump. The operating parameters ($p = 5\ Torr$, $Q_{N_2} = 1.0\ slpm$, $P_{MW} = 200\ W$, $d = 56\ cm$) were chosen in order to obtain pure late afterglow conditions (PLAC) in the treatment reactor. With such conditions, a gas temperature slightly higher than the

room temperature ($T = 30\,^{\circ}\mathrm{C}$) and a high concentration of N atoms ($[N] = 1.7\ 10^{21} m^{-3}$) were measured in the treatment chamber, using optical emission spectroscopy [3].

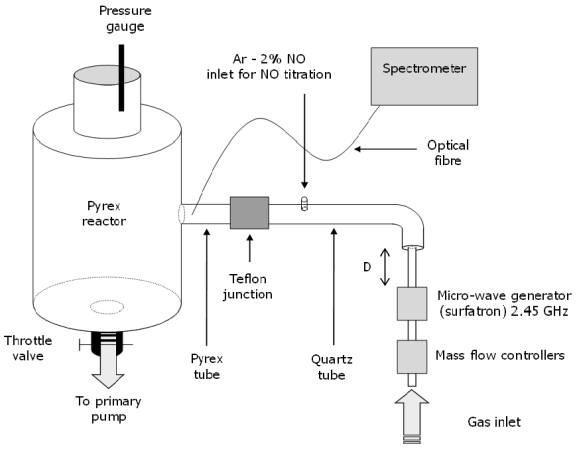

Figure 1: *Flowing afterglow set up*

2.b. Microbiological protocol

To study the bacterial inactivation we used an *Escherichia coli* strain (CIP: 54.8 T) obtained from the International Collection of Pasteur. After an overnight incubation in a *Luria-Bertani* (LB) broth at $37\,^{\circ}\mathrm{C}$, bacteria were separated from the broth by centrifugation ($10\ min, 4000\ rpm$) and immersed in pure distilled water. A $10\ \mu l$ droplet, containing an average bacteria concentration of $10^7 - 10^8/ml$ was deposited on a sterile glass sheet, placed in the treatment chamber in an open Petri dish and slowly desiccated by a vacuum exposure ($15\ min$ at $0.01\ Torr$). After afterglow treatment, the bacterial film containing living and inactivated bacteria was immersed in $1\ ml$ of LB and bacteria retrieved by $90\ s$ gentle mechanical agitation. $100\ \mu l$ of the recovery suspension were taken, eventually diluted and spread on agar in Petri dishes. The colonies formed from the surviving bacteria were finally numbered after $24\ h$ of incubation at $37\,^{\circ}\mathrm{C}$.

2.c. Lipid A quantification

A solution of lipid A was prepared by sonication ($1\ mg/ml$) in H_2O and was used as standard for quantification experiments. For this purpose, $1\ \mu g$ of P-LA was desiccated on a sterile glass slide, resulting in a thin film of $5\ mm$ in diameter, and was submitted during $40\ min$ to the pure nitrogen afterglow, in the same conditions as bacteria (see in 2.b.). At the end, lipid A was eluted from the microscope slides by $500\ \mu l$ of a chloroform/methanol/H_2O solution twice. The chloroformic phase was dried under nitrogen, suspended in $20\ \mu l$ of solvent mix, bath sonicated for $5\ min$ and spotted on a nitrocellulose membrane. Membranes were blocked with a Tris-Nacl solution, containing $0,1\%$ Tween 20 , 10% non fat dry milk, for *1 h* at room temperature. Then, membranes were incubated overnight at $4\,^{\circ}\mathrm{C}$ with an anti-lipid A goat primary antibody using a dilution of $1/400$ in 1% non fat milk TBST. A second incubation was done with an anti-goat immunoglobuli horseradish peroxydase coupled secondary antibody at a dilution of $1/5000$ for *1 h* at room temperature. After several washes, dots were detected using western blotting detection reagents. Relative intensity of each spot was scanned and quantified using Image J software. Controls without afterglow exposure were done in the same conditions. The relative B-LA (vacuum-treated and vacuum + afterglow exposed) was evaluated vs a dot-blot calibration curve of different lipid A concentrations ranging from 0.25 to $2\ \mu g$. Alternatively, the B-LA of control and afterglow-treated bacteria was estimated in the following conditions: approximatively $10\ \mu l$ of bacteria suspension (average bacteria concentration of $(10^7 - 10^8/ml)$ were spotted on glass slides and exposed to the afterglow, as described in Bacteria were collected in water and lipid A was extracted by addition of a chloroform/methanol/H_2O mix . As previously described for native lipid A, the solvent phase of samples were evaporated under nitrogen, suspended in $100\ \mu g$ of mix and bath sonicated for $5\ min$. An aliquot of $5\ \mu g$ was spotted on nitrocellulose membrane and the B-LA was detected by Dot-blot, as above indicated.

2.d. Nuclear translocation of the NF-κB transcription factor

The inflammatory effect of lipid A was investigated in murine aortic endothelial cells were grown in $100\ mm$ culture dishes, in DMEM containing $Glutamax^{®}$ and supplemented with 10% fetal calf serum (FCS), penicillin ($100\ units/mL$) and streptomycin ($100\ \mu g/mL$), as previously used in [6]. At sub-confluency, the medium was removed and replaced by fresh FCS free-DMEM medium. After $24\ h$, the cells were stimulated for $20\ min$ by lipid A ($200\ ng/ml$), untreated or vacuum-treated, and by an identical volume of

afterglow-treated residual lipid A, in 0.5% FCS DMEM medium. A positive control was done using TNF-α ($20\ ng/ml,\ 20min$). In order to test the change of proinflammatory potential of bacteria, vacuum or afterglow-treated bacteria were collected in $100\ \mu l$ of water and bath sonicated for $10\ min$. Then $10\ \mu l$ of bacteria homogenates were incubated with cells for $2\ hours$. At the end, the cells were washed $3\ times$ in phosphate-buffered saline and the nuclei (containing the activated *NF-κB* transcription factor) were extracted using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Pierce), according to the manufacturer's protocol. Protein concentration in the purified nuclei was determined using the Bradford reagent (Biorad). Equal amount ($25\ \mu g$) of nuclei extracts were loaded on a SDS-polyacarylamide gel and electrotransferred to polyvinylidene fluoride membrane, under the previously used conditions [6]. Immunoblotting was performed with a primary anti- *NF-κB* p65 antibody at the concentration of $0,5\ \mu g/ml$.

3. RESULTS

3.a. Effect of the UV irradiation

The role of UV radiation in the inactivation mechanisms of nitrogen late afterglow was checked by placing a MgF_2 filter as a lid on the open Petri dish containing the bacterial film. With a cutting wavelength at $120\ nm$, the MgF_2 filter allows the UV radiation at $320\ nm$ to interact with the bacteria, while considerably reducing the access of the active species of the afterglow.

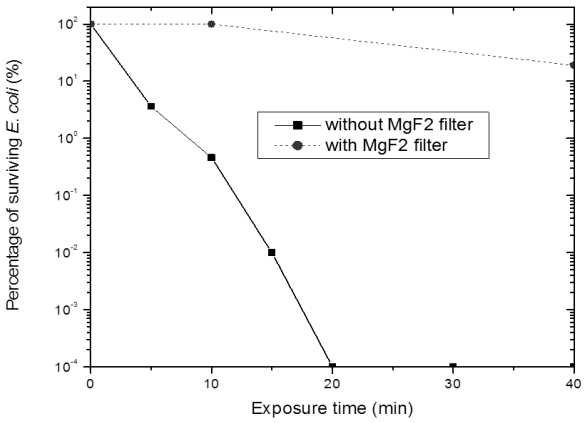

Figure 2: *Survival curves obtained for E. coli bacteria exposed in nitrogen PLAC with and without the MgF_2 filter.*

The survival curves obtained with and without the MgF_2 filter are given in Fig.2. With the MgF_2 filter, after $10\ min$ of exposure to the nitrogen afterglow, no significant inactivation is found while after $40\ min$ of exposure it is only obtained a $0.9\ log$ reduction of the initial E. coli population, much lower than the one obtained without filter, whatever the exposure time Fig.(2). This result demonstrates that the UV photons produced outside the Petri dish are not the main responsible for the E. coli inactivation. In consequence, either the UV interaction with the bacterial DNA is not the dominant inactivation mechanism of the nitrogen afterglow or only the photons produced by the atomic recombinations at the bacteria close vicinity (inside the Petri dish) are efficient.

3.b. Lipid A removal

We developped a dot blot method based on an immuno detection of lipid A in order to determine whether the afterglow exposure affects the lipid A content, in the exposed bacteria.

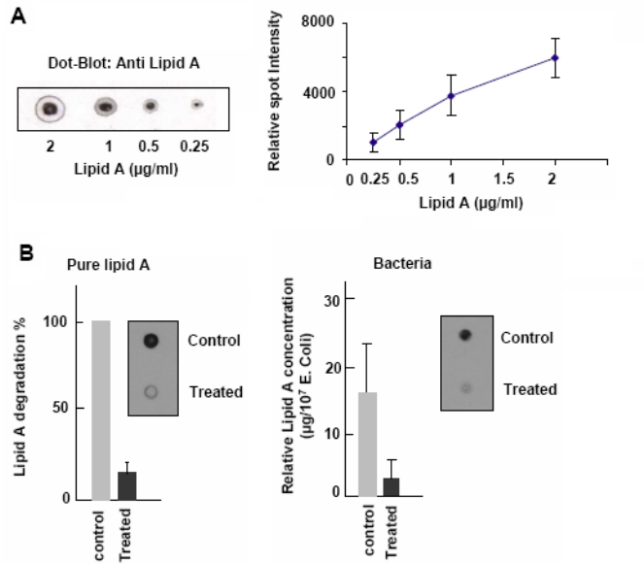

Figure 3: *Effect of nitrogen afterglow exposure on lipid A A. Dot blot binding assay: dose-response calibration curve represents increasing concentrations of lipid A spotted on nitrocellulose membranes and blotted with an anti lipid A antibody. B. pictures of P-LA dot-blots (left) or from B-LA (right), in vacuum-treated and vacuum + nitrogen afterglow treated conditions. Mean +/-SEM of 5 separate experiments, * < p.0.05.*

The residual lipid A content was quantified using a standard lipid A curve Fig.(3A) and compared to $1\ \mu g$ lipid A treated by vacuum (but not by nitrogen afterglow) in order to build a dose-response calibration curve. As shown in Fig.3B, the residual P-LA present on the slide after exposure to the nitrogen afterglow was less than 20% of the control, indicating that the afterglow treatment allowed to remove or degrade the endotoxin from

the treated slide. Then we analyzed the afterglow effects on B-LA. Lipid A was extracted from bacteria and analyzed by the same dot blot method. A decrease in B-LA was also observed, leading to the conclusion that lipid A localisation in plasmic membrane of bacteria was submitted to afterglow transformation. Nevertheless, high variability could be observed for quantification of B-LA matrix by the dot blot method. We then investigated whether the afterglow exposure affects the proinflammatory properties evoked by lipid A, and assessed by the nuclear translocation of the *NF-κB* transcription factor [5, 7, 8]. As shown in Fig.4A, $2\ h$ treatment of the murine aortic endothelial cells CRL2181, by untreated P-LA ($0.2\ \mu g/ml$) resulted in the nuclear translocation of *NF-κB*, as reported [8]. Same results were obtained in the presence of vacuum-treated P-LA.

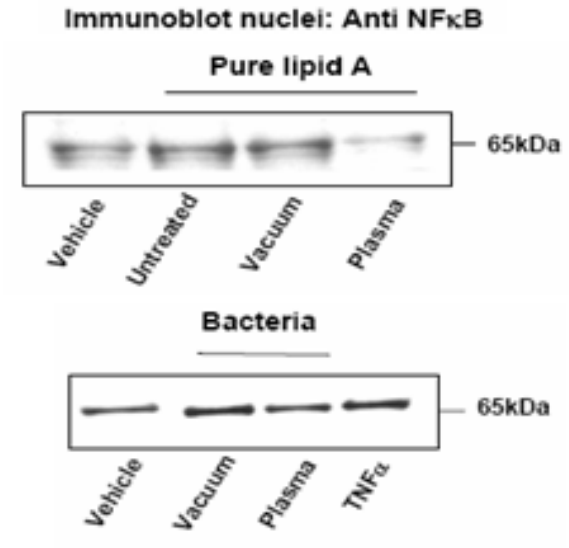

Figure 4: *Nuclear translocation of the NF-κB transcription factor In A, effect of P-LA: CRL2181 murine endothelial cells were treated for* $20\ min$ *with lipid A (*$200\ ng/ml$*) after low pressure treatment (vacuum), plasma treatment (*$5\ Torr, 200\ W, 40\ min$*) (plasma) or from stock solution (untreated). In B, effect of LA-B: CRL2181 were stimulated for* $2\ h$ *with* $10\ \mu l$ *of E. coli extracts obtained after treatment with low pressure (*$5\ Torr$*) for* $15min$ *and* N_2 *post-discharge at* $200\ W$ *for* $40\ min$*, or low pressure only, for bacteria control. The nuclei were extracted and used for immunoblotting of NF-κB as in A. These results are representative of 3 separate experiments*

In contrast, no nuclear translocation of *NF-κB* was observed in cells stimulated by a same volume of afterglow-exposed P-LA. Since the proinflammatory and toxic activity of gram-negative bacteria such as *E. coli* resides mainly in LPS, and more precisely in lipid A [8], we checked whether afterglow-treated bacteria may trigger the nuclear translocation of *NF-κB*, by comparison with control and vacuum-treated bacteria. As shown in Fig.4B, bacteria extracts stimulated the translocation of *NF-κB*, whereas nitrogen afterglow exposure inhibited this cellular response, indicating that the treatment by the afterglow strongly affects the biological activity of B-LA, probably by removing it from the support.

4. CONCLUSION

The *E. coli* viability reduction, is only correlated with the $N\ atom$ concentration of the late afterglow and not with the UV-C production, as demonstrated with MgF_2 experiments. Furthermore, we report a 80% loss P-LA ($1\ \mu g$ deposited at a concentration of $1\ mg/ml$) for a $40\ min$ exposure to the pure N_2 afterglow, comparable to the one obtained with LA-B. We also observed a net decrease of the proinflammatory activity of the exposed lipid A, assessed by the nuclear translocation of the redox-senstive transcription factor *NF-κB*. To conclude, this study confirms the interest of pure nitrogen afterglows as a potential sterilizing system that strongly affects *E. coli* viability and substantially removes the amount of lipid A present at the surface of the reusable medical instrumentation.

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